Evidence for the agricultural origin of antimicrobial resistance in a fungal pathogen of humans

Authors and affiliations

S. Earl Kang[1,3], Leilani G. Sumabat[2,4], Tina Melie[2,5], Brandon Mangum[1, 2], Michelle Momany[1*], Marin T. Brewer[2*]

[1]Fungal Biology Group & Plant Biology Department, University of Georgia, Athens, GA
[2]Fungal Biology Group & Plant Pathology Department, University of Georgia, Athens, GA
[3]Current address: Fungal Team, Ginkgo Bioworks, Boston, MA
[4]Current address: Institute of Biology, University of the Philippines Diliman, Quezon City, Philippines
[5]Current address: Ecology and Evolutionary Biology Department, University of Colorado, Boulder, CO

* Corresponding authors

Abstract

Resistance to clinical antimicrobials is an urgent problem, reducing our ability to combat deadly pathogens of humans. Azole antimicrobials target ergosterol synthesis and are highly effective against fungal pathogens of both humans and plants leading to their widespread use in clinical and agricultural settings[1,2]. The fungus *Aspergillus fumigatus* causes 300,000 life-threatening infections in susceptible human hosts annually and azoles are the most effective treatment[3]. Resistance to clinical azole antifungals has become a major problem in Europe and India over the last decade, where identical mutations in *cyp51A*, an ergosterol biosynthetic gene, have been found in strains from both clinical and agricultural settings[4]. Shared *cyp51A* genotypes suggest that clinical azole resistance might have had an agricultural origin; however, until now, independent origins of clinical and agricultural mutations could not be ruled out. Here we show that azole-resistant isolates of *A. fumigatus* from clinical and agricultural settings also carry mutations conferring resistance to quinone outside inhibitor (QoI) fungicides, which are used exclusively in agricultural settings. This is the first report of a clear marker for the agricultural origin of resistance to a clinical antifungal. We anticipate that our work will increase the understanding of interactions between pathogens of plants and pathogens of humans.

Main

Fungi are important pathogens of humans, causing over 1.5 million deaths annually[5]. Fungi are also important pathogens of plants, causing crop losses of 20% and postharvest losses of 10%[6]. The filamentous fungus *Aspergillus fumigatus* is a saprobe found in a variety of environments including soil, compost, and decaying plant material; however, in immunocompromised individuals it can cause the devastating disease aspergillosis. Azoles are often used as the first line of defense against aspergillosis. During the last decade Europe and Asia have seen an alarming increase in azole-resistant *A. fumigatus* in the clinic and azole resistance is now present on 6 continents[7]. Though some resistance has been associated with long-term azole therapy in patients with chronic infections, at least two-thirds of patients with azole-resistant *A. fumigatus* infections have not previously undergone azole therapy[8,9]. The environmental use of azoles has been proposed to be the driving force for the majority of clinical resistance in *A. fumigatus* with several studies suggesting that most azole-resistant isolates originated from widespread agricultural use of azoles to combat plant-pathogenic fungi[10,11].

The same mutations in *cyp51A* – which encodes the ergosterol biosynthetic protein targeted by azoles – have been reported in strains from both clinical and agricultural settings in Europe, Asia, Africa, and the Middle East[4,7,9,12]. Several point mutations and tandem repeats (TR) within the promoter, including TR_{34}/L98H and TR_{46}/Y121F/T289A, are commonly associated with azole resistance in environmental isolates. Isolates with the TR_{34}/L98H and TR_{46}/Y121F/T289A alleles show high levels of resistance to multiple azole drugs (pan-azole resistance) and patients infected with these isolates have higher rates of treatment failure and death[13]. Though the presence of TR_{34}/L98H and TR_{46}/Y121F/T289A alleles in both agricultural and clinical isolates suggested that azole-resistant clinical strains of *A. fumigatus* might have had an agricultural origin, independent evolution of these mutations in both settings has not been previously excluded.

In the Unites States (U.S.), azole-resistant *A. fumigatus* strains with TR_{34}/L98H and TR_{46}/Y121F/T289A alleles have been recently reported in patients[14,15]. Additionally, azole-resistant *A. fumigatus* isolates with TR_{34}/L98H alleles were detected in a peanut field in Georgia[16]. Otherwise, very little information about the frequency of azole-resistant *A. fumigatus* in agricultural settings in the U.S. is available. To investigate the prevalence of azole-resistant *A. fumigatus* in agricultural environments in the U.S., we collected soil and plant debris from 56 sites across Georgia and Florida, including 53 sites with a history of azole fungicide use, two organic sites with no fungicide use for at least 10 years, and one compost pile of unknown history (Supplementary Table S1). We recovered 700 isolates of *A. fumigatus* from soil, plant debris, and compost. Isolates were screened for sensitivity to the azole fungicide tebuconazole (TEB) that has widespread use in agriculture. Of the 700 isolates collected, 123 (17.6%) grew on solid medium amended with 3 μg/ml TEB. None of the isolates collected from the two organic sites grew on TEB-amended plates.

Minimal Inhibitory Concentrations (MIC) for TEB, itraconazole (ITC), voriconazole (VOR), and posaconazole (POS) were determined by broth dilution assays for the 123 isolates that grew on TEB-amended solid medium, and for 49 isolates from the same sites that that did not grow on the amended medium. MIC ranged from 0.5 to > 16 μg/ml for TEB, 0.5 to 2 μg/ml for ITC, 0.125 to > 16 μg/ml for VOR, and 0.06 to 1 μg/ml for POS. Recommended clinical breakpoints of antifungal resistance for *A. fumigatus*[17] are > 2 μg/ml for ITC and VOR and > 0.25 μg/ml for POS; however, EUCAST notes there is uncertainty regarding the cutoff values for POS and some data suggest the cutoff value of > 1 μg/ml, which we use here, may be more relevant. Although many of the isolates showed low levels of azole resistance, only 12 of the 123 isolates were highly resistant at clinically relevant levels (Supplementary Tables S1 and S2). The 12 isolates exhibited high levels of resistance to both TEB and VOR with MIC ≥ 16 μg/ml, and decreased sensitivity to ITC and POS (Table 1), showing they are pan-azole resistant.

To determine whether mutations in *cyp51A* were associated with azole resistance, we sequenced 1,286 bp of *cyp51A*, including the promoter and downstream regions, for 123 isolates that grew on TEB-amended medium and for 49 TEB-sensitive isolates from the same sites. The 12 pan-azole-resistant isolates had the TR_{46}/Y121F/T289A allele (Table 1) that underlies high levels of resistance to VOR[10,12]. We did not detect the TR_{34}/L98H allele that is the most prevalent worldwide in azole-resistant environmental and clinical isolates of *A. fumigatus*. Failure to detect the TR_{34}/L98H allele does not necessarily mean this set of mutations is absent in the areas we sampled, but more likely reflects our initial screen for resistance with TEB. TEB is an azole with a similar structure to VOR, and resistance to TEB has previously been associated with the TR_{46}/Y121F/T289A mutations[18]. Eleven of the isolates sequenced had the I242V mutation in Cyp51A and 5 had the Y46F/V172M/T248N/E255D/K427E mutations found in the reference isolate Af293. These 16 isolates with non-synonymous mutations in *cyp51A,* but without tandem repeats in the promoter, had slightly elevated MIC values for TEB, VOR, and POS compared to the sensitive reference isolate A1163 (Table 1). Increased MIC values for isolates with these mutations have been described previously[19-21]; however, it is not clear if these specific mutations are the cause of increased drug resistance.

To determine the relationship of agricultural isolates from Georgia and Florida to clinical isolates from the same region, we used 9 STR*Af* markers[22] to genotype the 168 agricultural isolates that we collected along with 48 clinical isolates collected between 2015 and 2017 by the Centers for Disease Control and Prevention[23]. None of the clinical isolates were azole resistant. Based on microsatellite data almost every isolate had a unique genotype (Supplementary Figure S1). The combined environmental and clinical *A. fumigatus* population from Georgia and Florida showed no genetic structure, except for the pan-azole-resistant agricultural isolates that had the TR_{46}/Y121F/T289A allele. These pan-azole-resistant isolates comprised a single lineage and were isolated from a compost pile and pecan debris from a processing facility (Supplementary Table S1). Our results are consistent with previous studies showing that *A. fumigatus* is panmictic with little population structure either by geographic region or clinical versus agricultural setting[9,24].

To better understand the relationship of Georgia and Florida agricultural isolates to worldwide environmental and clinical isolates, we performed whole genome sequencing on 89 strains representing all of our field sites and combined them with 69 publicly available whole genome sequences to construct a neighbor-joining tree (Figure 1; Supplementary Table S3). Clinical and environmental azole-resistant isolates (open or closed red circles) from the United States (USA), United Kingdom (UK), Spain (ESP), the Netherlands (NL), and India (IND) were distributed throughout the tree; however, the four pan-azole-resistant isolates from this study that carried *cyp51A* TR mutations

(closed red circles) clustered into a well-supported clade with clinical and environmental isolates carrying *cyp51A* TR mutations from the UK, IND and NL (red branches). This clade also included azole-sensitive isolates from diverse geographic origins. Although *A. fumigatus* does not show population structure by geographic or environmental origin, our data support population structure by pan-azole resistance. The genetic relatedness of pan-azole-resistant isolates across geographic locations and environment types has been previously described[9,24] and suggests that there is a barrier to gene flow or some other genetic predisposition in this pan-azole-resistant clade allowing *cyp51A* TR mutations to arise and/or persist.

To delay the evolution and spread of antifungal resistance in agricultural settings, azoles are generally applied to crops in alternation or combination with other fungicides such as the quinone outside inhibitors (QoI) and, to a lesser extent, benzimidazoles (MBC) [25,26]. QoI fungicides target the protein encoded by the *cytochrome B* (*cytB*) gene and are used on crops, but not on patients[27]. MBC fungicides target the protein encoded by the *ß-tubulin* (*benA*) gene and were widely used in U.S. agriculture in the 1970s. MBCs are used much less in U.S. agriculture now due to resistance development, but they are used clinically as antihelminthics[28]. We reasoned that if isolates of *A. fumigatus* had acquired azole-resistance in agricultural settings, they might also have acquired resistance to these non-azole fungicides. To determine if azole-resistant isolates carried mutations conferring resistance to QoI and MBC fungicides, we searched the genomes of our agricultural isolates along with the genomes of publicly available *A. fumigatus* isolates (Supplementary Table S3). We detected the *cytB* G143A mutation known to cause QoI resistance (Figure 1, orange circles; Table 2) and/or the *benA* F219Y mutation known to cause MBC resistance (Figure 1, violet circles; Table 2) in 14 clinical and environmental isolates, including four from Georgia agricultural sites. To verify that these mutations were associated with fungicide resistance, we tested growth on media amended with QoI or MBC fungicides for the four Georgia isolates carrying *cytB* and *benA* mutations (eAF222, eAF233, eAF234, eAF513) and four Georgia isolates not carrying these mutations (eAF77, eAF94, eAF128, eAF537). Only isolates with the *cytB* G143A mutation grew on medium with the QoI fungicide azoxystrobin and only isolates with the *benA* F219Y mutation grew on medium with the MBC fungicide benomyl (Supplementary Figure S2, Supplementary Table S4).

Fourteen of 19 pan-azole-resistant isolates included in our study also carried mutations for QoI resistance, MBC resistance, or both. All QoI-resistant and MBC-resistant mutants were also pan-azole resistant and clustered in the well-supported pan-azole clade (Figure 1, red branches). Eight of these multi-fungicide-resistant isolates were from agricultural sources in the United States and India and 6 were from clinical sources in the Netherlands and India. Four of these clinical isolates carried mutations conferring resistance to both MBC and QoI fungicides. That pan-azole-resistant *A. fumigatus* strains from patients carry the mutation for resistance to QoI fungicides used exclusively for plant protection shows definitively that these strains have an agricultural origin. However, not all pan-azole resistant isolates in our study showed this definitive signature of agricultural origin. Five of 19 pan-azole-resistant clinical isolates did not carry MBC or QoI fungicide-resistance mutations raising the possibility that they could have originated from nonagricultural sources. We found pan-azole-resistant *A. fumigatus* in a single clade on three continents. We also found resistance to MBC and QoI fungicides exclusively in this clade and always in combination with pan-azole resistance. Beyond showing the agricultural origin of clinical pan-azole resistance, our results suggest that the unique genetics of the pan-azole clade enable the evolution and/or persistence of antimicrobial resistance mutations.

References

1 Klittich, C. J. Milestones in Fungicide Discovery: Chemistry that Changed Agriculture. *Plant Health Progress* **9**, 31 (2008).

2 Sheehan, D. J., Hitchcock, C. A. & Sibley, C. M. Current and emerging azole antifungal agents. *Clin Microbiol Rev* **12**, 40-79 (1999).

3 Patterson, T. F. *et al.* Executive Summary: Practice Guidelines for the Diagnosis and Management of Aspergillosis: 2016 Update by the Infectious Diseases Society of America. *Clin Infect Dis* **63**, 433-442, doi:10.1093/cid/ciw444 (2016).

4 Verweij, P. E., Chowdhary, A., Melchers, W. J. & Meis, J. F. Azole Resistance in Aspergillus fumigatus: Can We Retain the Clinical Use of Mold-Active Antifungal Azoles? *Clin Infect Dis* **62**, 362-368, doi:10.1093/cid/civ885 (2016).

5 Brown, G. D. *et al.* Hidden killers: human fungal infections. *Sci Transl Med* **4**, 165rv113, doi:10.1126/scitranslmed.3004404 (2012).

6 Fisher, M. C., Hawkins, N. J., Sanglard, D. & Gurr, S. J. Worldwide emergence of resistance to antifungal drugs challenges human health and food security. *Science* **360**, 739-742, doi:10.1126/science.aap7999 (2018).

7 Resendiz Sharpe, A. *et al.* Triazole resistance surveillance in Aspergillus fumigatus. *Med Mycol* **56**, 83-92, doi:10.1093/mmy/myx144 (2018).

8 Bowyer, P. & Denning, D. W. Environmental fungicides and triazole resistance in Aspergillus. *Pest Manag Sci* **70**, 173-178, doi:10.1002/ps.3567 (2014).

9 Snelders, E. *et al.* Possible environmental origin of resistance of Aspergillus fumigatus to medical triazoles. *Appl Environ Microbiol* **75**, 4053-4057, doi:10.1128/AEM.00231-09 (2009).

10 Berger, S., El Chazli, Y., Babu, A. F. & Coste, A. T. Azole Resistance in Aspergillus fumigatus: A Consequence of Antifungal Use in Agriculture? *Front Microbiol* **8**, 1024, doi:10.3389/fmicb.2017.01024 (2017).

11 Chowdhary, A., Kathuria, S., Xu, J. & Meis, J. F. Emergence of azole-resistant aspergillus fumigatus strains due to agricultural azole use creates an increasing threat to human health. *PLoS Pathog* **9**, e1003633, doi:10.1371/journal.ppat.1003633 (2013).

12 Moore, C. B. *et al.* First isolation of the pan-azole-resistant Aspergillus fumigatus cyp51A TR46/Y121F/T289A mutant in a UK patient. *Int J Antimicrob Agents* **49**, 512-514, doi:10.1016/j.ijantimicag.2017.01.004 (2017).

13 van der Linden, J. W. *et al.* Clinical implications of azole resistance in Aspergillus fumigatus, The Netherlands, 2007-2009. *Emerg Infect Dis* **17**, 1846-1854, doi:10.3201/eid1710.110226 (2011).

14 Wiederhold, N. P. *et al.* First Detection of TR34 L98H and TR46 Y121F T289A Cyp51 Mutations in Aspergillus fumigatus Isolates in the United States. *J Clin Microbiol* **54**, 168-171, doi:10.1128/JCM.02478-15 (2016).

15 Vazquez, J. A. & Manavathu, E. K. Molecular Characterization of a Voriconazole-Resistant, Posaconazole-Susceptible Aspergillus fumigatus Isolate in a Lung Transplant Recipient in the United States. *Antimicrob Agents Chemother* **60**, 1129-1133, doi:10.1128/AAC.01130-15 (2016).

16 Hurst, S. F., Berkow, E. L., Stevenson, K. L., Litvintseva, A. P. & Lockhart, S. R. Isolation of azole-resistant Aspergillus fumigatus from the environment in the south-eastern USA. *J Antimicrob Chemother* **72**, 2443-2446, doi:10.1093/jac/dkx168 (2017).

17 EUCAST. *Breakpoint tables for interpretation of MICs meters, version 9.0.* (The European Committee on Antimicrobial Susceptibility Testing, 2018).

18 Chowdhary, A., Sharma, C., Kathuria, S., Hagen, F. & Meis, J. F. Azole-resistant Aspergillus fumigatus with the environmental TR46/Y121F/T289A mutation in India. *J Antimicrob Chemother* **69**, 555-557, doi:10.1093/jac/dkt397 (2014).

19 Pham, C. D., Reiss, E., Hagen, F., Meis, J. F. & Lockhart, S. R. Passive surveillance for azole-resistant Aspergillus fumigatus, United States, 2011-2013. *Emerg Infect Dis* **20**, 1498-1503, doi:10.3201/eid2009.140142 (2014).

20 Howard, S. J. *et al.* Frequency and evolution of Azole resistance in Aspergillus fumigatus associated with treatment failure. *Emerg Infect Dis* **15**, 1068-1076, doi:10.3201/eid1507.090043 (2009).

21 Rivero-Menendez, O., Alastruey-Izquierdo, A., Mellado, E. & Cuenca-Estrella, M. Triazole Resistance in Aspergillus spp.: A Worldwide Problem? *J Fungi (Basel)* **2**, doi:10.3390/jof2030021 (2016).

22 de Valk, H. A. *et al.* Use of a novel panel of nine short tandem repeats for exact and high-resolution fingerprinting of Aspergillus fumigatus isolates. *J Clin Microbiol* **43**, 4112-4120, doi:10.1128/JCM.43.8.4112-4120.2005 (2005).

23 Berkow, E. L. *et al.* Detection of TR34/L98H CYP51A Mutation through Passive Surveillance for Azole-Resistant Aspergillus fumigatus in the United States from 2015 to 2017. *Antimicrob Agents Chemother* **62**, doi:10.1128/AAC.02240-17 (2018).

24 Abdolrasouli, A. *et al.* Genomic Context of Azole Resistance Mutations in Aspergillus fumigatus Determined Using Whole-Genome Sequencing. *mBio* **6**, e00536, doi:10.1128/mBio.00536-15 (2015).

25 Hobbelen, P. H., Paveley, N. D., Oliver, R. P. & van den Bosch, F. The usefulness of fungicide mixtures and alternation for delaying the selection for resistance in populations of Mycosphaerella graminicola on winter wheat: a modeling analysis. *Phytopathology* **103**, 690-707, doi:10.1094/PHYTO-06-12-0142-R (2013).

26 FRAC. *FRAC recommendations for fungicide mixtures designed to delay resistance evolution.* . (Fungicide Resistance Action Committee, 2010).

27 Fernández-Ortuño, D., Torés, J. A., De Vicente, A. & Pérez-García, A. The QoI Fungicides, the Rise and Fall of a Successful Class of Agricultural Fungicides in *Fungicides* (IntechOpen, 2010).

28 Furtado, L. F. V., de Paiva Bello, A. C. P. & Rabelo, E. M. L. Benzimidazole resistance in helminths: From problem to diagnosis. *Acta Trop* **162**, 95-102, doi:10.1016/j.actatropica.2016.06.021 (2016).

223 **TABLE 1.** Azole-susceptibility of environmental *A. fumigatus* isolates with non-synonymous *cyp51A* mutations.
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Isolates	*cyp51A* genotype [a]	MIC Ranges (μg/ml) [b]			
		TEB	ITC	VOR	POS
A1163	WT	1.0	1.0	0.25	0.25
eAF221	TR_{46}/Y121F/T289A	>16	1.0-2.0	>16	1.0
eAF222	TR_{46}/Y121F/T289A	>16	2.0	>16	1.0
eAF228	TR_{46}/Y121F/T289A	>16	1.0-2.0	>16	1.0
eAF229	TR_{46}/Y121F/T289A	>16	1.0-2.0	>16	1.0
eAF230	TR_{46}/Y121F/T289A	>16	1.0-2.0	>16	1.0
eAF231	TR_{46}/Y121F/T289A	>16	1.0-2.0	>16	1.0
eAF232	TR_{46}/Y121F/T289A	>16	1.0-2.0	>16	1.0
eAF233	TR_{46}/Y121F/T289A	>16	1.0-2.0	>16	1.0
eAF234	TR_{46}/Y121F/T289A	>16	1.0-2.0	>16	1.0
eAF235	TR_{46}/Y121F/T289A	16	1.0	≥16	0.5-1.0
eAF236	TR_{46}/Y121F/T289A	>16	1.0-2.0	>16	1.0
eAF513	TR_{46}/Y121F/T289A	>16	1.0	>16	1.0
eAF010	I242V	1.0-2.0	1.0	0.5	0.5-1.0
eAF175	I242V	2.0	1.0	0.25-0.5	0.5
eAF263	I242V	2.0	1.0	0.5	0.5
eAF265	I242V	2.0	1.0	0.5	0.5
eAF406	I242V	2.0	1.0	0.5	0.25
eAF500	I242V	2.0	1.0	0.5	0.5
eAF589	I242V	2.0	1.0	0.25-0.5	0.25-0.5
eAF621	I242V	2.0	1.0	0.5	0.5
eAF647	I242V	2.0	1.0	0.25	0.25
eAF773	I242V	2.0	1.0	0.5	0.5
eAF792	I242V	1.0-2.0	0.5-1.0	0.25-0.5	0.25-0.5
eAF016	Y46F/V172M/T248N/ E255D/K427E	1.0-2.0	1.0	0.25-0.5	0.5
eAF082	Y46F/V172M/T248N/ E255D/K427E	1.0-2.0	1.0	0.25-0.5	0.25-0.5
eAF128	Y46F/V172M/T248N/ E255D/K427E	2.0	1.0	0.5	0.5
eAF163	Y46F/V172M/T248N/ E255D/K427E	1.0-2.0	1.0	0.25-0.5	0.5
eAF609	Y46F/V172M/T248N/ E255D/K427E	2.0-4.0	1.0	0.5-1.0	0.25-0.5

225 [a] Cyp51A protein GenBank accession number EDP50065.1 used as reference. [b] MICs, Minimal Inhibitory
226 Concentration; TEB, tebuconazole; ITC, itraconazole; VOR, voriconazole; POS, posaconazole. Each assay was
227 conducted twice.
228
229

TABLE 2. Mutations associated with fungicide resistance in pan-azole-resistant *A. fumigatus*.

Isolate	Source	*cyp51A* – azoles [a]	*cytB* – QoI [b]	*benA* – MBC [c]
A1163	Clinic	WT	WT	WT
eAF222	Environment, 2018 USA	TR_{46}/Y121F/T289A	G143A	F219Y
eAF233	Environment, 2018 USA	TR_{46}/Y121F/T289A	G143A	F219Y
eAF234	Environment, 2018 USA	TR_{46}/Y121F/T289A	G143A	F219Y
eAF513	Environment, 2018 USA	TR_{46}/Y121F/T289A	G143A	F219Y
08-12-12-13	Clinic, 2003 Netherlands	TR_{34}/L98H/	WT	F219Y
08-31-08-91	Clinic, 2004 Netherlands	TR_{34}/L98H	WT	F219Y
08-36-03-25	Clinic, 2005 Netherlands	TR_{34}/L98H	WT	F219Y
10-01-02-27	Clinic, 2010 Netherlands	TR_{34}/L98H	G143A	F219Y
Afu 942/09	Clinic, 2009 India	TR_{34}/L98H	G143A	F219Y
Afu 1042/09	Clinic, 2009 India	TR_{34}/L98H	G143A	F219Y
Afu 124/E11	Environment, 2011 India	TR_{34}/L98H	G143A	F219Y
Afu 166/E11	Environment, 2011 India	TR_{34}/L98H	G143A	F219Y
Afu 218/E11	Environment, 2011 India	TR_{34}/L98H	G143A	F219Y
Afu 257/E11	Environment, 2011 India	TR_{34}/L98H	G143A	F219Y
Afu 343/P11	Clinic, 2011 India	TR_{34}/L98H	G143A	F219Y
Afu 591/12	Clinic, 2012 India	TR_{34}/L98H	G143A	F219Y
DI 15-96	Clinic, 2008 USA	TR_{46}/Y121F/T289A	G143A	F219Y
DI 15-102	Clinic, 2010 USA	TR_{34}/L98H	WT	F219Y
DI 15-106	Clinic, 2012 USA	TR_{46}/Y121F/T289A	G143A	F219Y
DI 15-116	Clinic, 2014 USA	TR_{34}/L98H	WT	F219Y

[a] GenBank accession number EDP50065 from azole-sensitive isolate A1163 was used as wildtype for Cyp51A. Isolates 08-12-12-13 and 08-36-03-25 also carried S297T/F495I mutations for Cyp51A, but these have not been associated with azole resistance.

[b] GenBank accession number YP_005353050 from azole-sensitive isolate A1163 was used as wildtype for CytB. All *cyp51A* TR mutants also carried V13I/I119V mutations for CytB, but these have not been associated with QoI resistance.

[c] GenBank accession number EDP56324 from azole-sensitive isolate A1163 was used as wildtype for BenA.

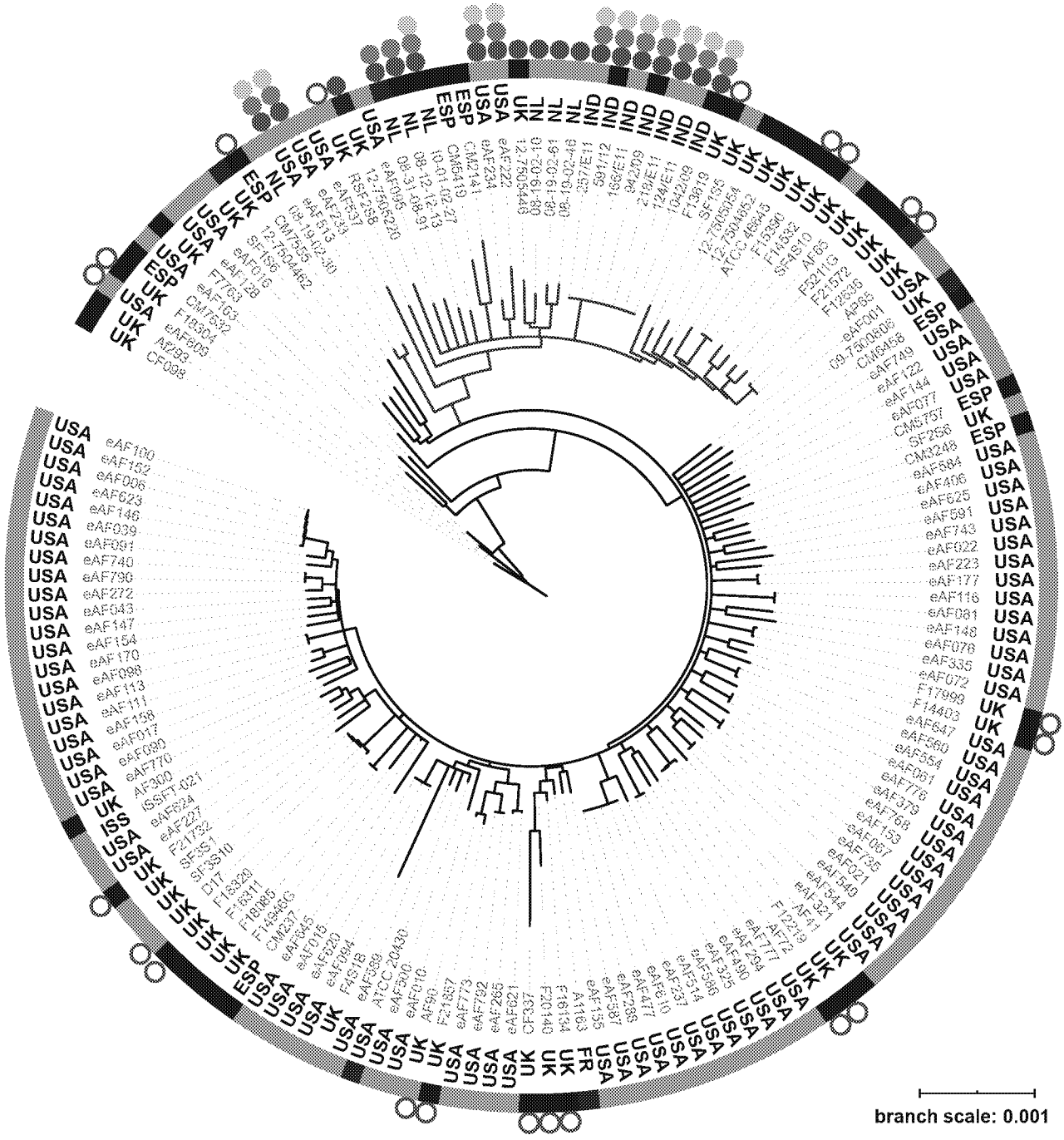

Figure 1. Neighbor-joining tree of environmental and clinical isolates of *Aspergillus fumigatus.* Whole genome sequences from Georgia and Florida agricultural sites (this study, eAFXXX), were analyzed along with publicly available data (Table S3). Af293 was used as the reference genome. Country of origin is listed next to each isolate (ESP=Spain, FR=France, IND=India, ISS=International Space Station, NL=Netherlands, UK=United Kingdom, USA=United States). Only branches with 100% bootstrap support based on 100 replicates are shown. Green bars indicate environmental isolates. Blue bars indicate clinical isolates. Solid red circles indicate pan-azole-resistant isolates with *cyp51A* TR mutations. Open red circles indicate azole-resistant isolates without TR mutations. Orange circles indicate isolates with *cytB* G143A mutation conferring resistance to QoI fungicides. Violet circles indicate *benA* F219Y mutation conferring resistance to MBC fungicides. Red branches indicate well-supported pan-azole-resistant clade.

Methods

Sampling

Between July 2017 and March 2018 we collected soil, plant debris, or compost from 56 agricultural sites in Georgia or Florida, USA, including 53 sites that had recently been treated with triazole fungicides, two sites that were in organic production with no triazole use in at least 10 years, and one compost pile with an unknown history of fungicide use on the plant material prior to composting (Supplementary Table S1). Each site was defined as a different field location, different crop at the same field location, or different triazole fungicide treatment. When soil was sampled, plant debris on the soil surface was included if available. If larger piles of debris were present on the soil surface they were collected separately. Soil was sampled by taking 5-10 soil cores to a depth of approximately 10 cm. Plant debris was sampled from the soil surface, cull piles at farms, and waste piles at pecan processing facilities. For each site we collected 4 samples at separate locations to minimize the collection of clones. Samples were stored in sealed plastic bags for transport and stored open to allow for gas exchange at 4°C for 2-20 d.

Isolation and storage

The samples were processed as described previously by Snelder et al.[9] and Hurst et al.[16] with some modifications. Briefly, 2 g of soil was suspended in 15 ml of sterile 0.1 M sodium pyrophosphate. Samples were vortexed for 30 s and allowed to settle for 1 min. From the supernatant, 100 µl was pipetted onto Sabouraud dextrose agar (SDA) in 100-mm Petri dishes supplemented with 50 µg/ml chloramphenicol (Sigma Aldrich) and 5 µg/ml gentamicin (Research Products International). The dishes were incubated for 2 to 4 d at 45°C. Colonies of *A. fumigatus* were initially identified based on morphology and screened for azole resistance on SDA supplemented with 3 µg/ml tebuconazole (TEB; Bayer Corp). Many of the isolates that grew on 3 µg/ml TEB-amended solid medium were not able to grow at similar concentrations of TEB in liquid medium during the MIC testing described below; therefore, these isolates are designated as sensitive to TEB in Supplementary Table S1. Isolates were stored at -80°C in 15% glycerol.

Antifungal susceptibility testing by Minimum Inhibitory Concentrations (MIC)

One hundred-seventy-two environmental *A. fumigatus* isolates (Supplementary Table S1), and 48 clinical isolates were tested for antifungal susceptibility under conditions described in the Clinical Laboratory Standard Institute broth microdilution method[29]. Antifungals tested included tebuconazole (TEB; TCI America, Oregon, USA), itraconazole (ITC; Thermo Sci Acros Organics, New Jersey, USA), voriconazole (VOR; Thermo Sci Acros Organics, New Jersey, USA), and posaconazole (POS; Apexbio Technology, Texas, USA) suspended in DMSO. Briefly, isolates were grown on complete media[30] slants for 5 to 7 d at 37°C and harvested with 2 ml of 0.02% Tween-20 solution. Spore suspensions were adjusted to 0.09 – 0.13 OD at 530 nm using a spectrophotometer. One-hundred microliters of 2×10^4 to 5×10^4 conidia/ml were added to 100 ml of RPMI 1640 liquid medium (Thermo Sci Gibco, California, USA) in microtiter plates with final concentrations of antifungals ranging from 0.015 to 16 µg/ml. Plates were incubated at 37°C for 48 h and MIC break points were determined visually. MIC break point was defined as the lowest concentration at which there was 100% inhibition of growth. Assays were repeated for all resistant isolates and most sensitive isolates. For classification of sensitivity or resistance for ITC and VOR, we used the recommended clinical breakpoints of antifungal resistance for *A. fumigatus*[17] which are > 2 µg/ml; however, EUCAST notes there is uncertainty regarding the cutoff values for POS and some data suggest > 1 µg/ml, which we use here, may be more relevant.

DNA extraction

High molecular weight genomic DNA of *A. fumigatus* isolates was extracted using a modified CTAB protocol as described previously[31]. Briefly, approximately 100 mg of mycelium collected from cultures that had been incubated overnight in liquid complete medium[30] were transferred to 2 ml tubes containing approximately 200 µl of 0.5-mm disruption glass beads (RPI, catalog #9831) and three 3-mm steel beads and lyophilized. Lyophilized cells were pulverized using Geno/Grinder at 1750 rpm for 30 s. Pulverized tissue was incubated in 1 ml of CTAB lysis buffer (100 mM Tris pH 8.0, 10 mM EDTA, 1% CTAB, 1% BME) for 30 min at 65°C. The samples were extracted with chloroform (500 µl) twice and DNA in the upper layer was precipitated in ice cold isopropanol. The precipitated DNA samples were washed with 70% ethanol twice, air dried, and dissolved in 100 µl sterile water. DNA was quantified using NanoDrop One (Thermo Sci, New Jersey, USA).

306 **cyp51A *sequencing***
307 For all environmental isolates in this study, *cyp51A* was PCR-amplified using previously designed primers[32]. PCR
308 reactions were performed with the Q5 Hot Start High-Fidelity 2× Master Mix (New England Biolabs) with 100 ng of
309 genomic DNA, 0.5 µM forward primer 5′-CGGGCTGGAGATACTATGGCT-3′ and 0.5 µM reverse primer 5′-
310 GTATAATACACCTATTCCGATCACACC-3′ in 20 µl reactions. PCR reactions were performed at 98°C for 2 min
311 followed by 30 cycles of 98°C for 15 s, 62°C for 15 s, and 72°C for 2:30 min, followed by a final extension at 72°C
312 for 5 min. Amplicons were sequenced by the Sanger method (Eurofins genomics, USA) using primers 5′-
313 GCATTCTGAAACACGTGCGTAG-3′, 5′-GTCTCCTCGAAATGGTGCCG-3′, and 5′-
314 CGTTCCAAACTCACGGCTGA-3′. Promoter sequences were aligned to A1163 genomic sequence v43 from
315 Ensembl. Coding sequences were translated to protein sequences and aligned to the Cyp51A protein of *A. fumigatus*
316 A1163 (GenBank accession EDP50065). Sequence analysis was performed using Geneious v11.1.5 (Biomatters,
317 Auckland, NZ).
318
319 ***Microsatellite genotyping***
320 Nine microsatellite markers (STR*Af* 2A, 2B, 2C, 3A, 3B, 3C, 4A, 4B, and 4C) previously developed for *A. fumigatus*
321 (de Valk et al. 2005) were used to genotype 166 environmental isolates (Supplementary Table 1), the reference
322 isolate Af293, and 48 clinical isolates from Georgia or Florida provided by the Mycotic Diseases Group at CDC[23].
323 Multiplex PCR was performed using the Type-it Microsatellite PCR kit (Qiagen) following the manufacturer's
324 protocol, but with the reaction volume modified to 10 µl. Multiplex reactions contained the following: 5 µl of 2×
325 Type-it Master Mix, 1 µl of 10× primer mix (2 µM of each primer in the multiplex), 1 µl of DNA template, and
326 RNAse-free water. Thermal cycling conditions had an initial denaturation at 95°C for 5 min followed by: 28 cycles
327 of 95°C for 30 s, 57°C for 90 s, and 72°C for 30 s and a final elongation of 60°C for 30 min. Amplification of a
328 product was confirmed by electrophoresis on a 1% (w/v) agarose gel with 1× TBE buffer. PCR products were diluted
329 (1:15) then 1 µl of diluted PCR product was added with 0.1 µl of the internal size standard Genescan-500 Liz
330 (Applied Biosystems) and 9.9 µl of Hi-Di formamide (Applied Biosystems). These were then incubated for 5 min at
331 95°C and placed immediately on ice. Fragment analysis was performed at the Georgia Genomics and Bioinformatics
332 Core (GGBC) on an Applied Biosystems 3730x1 96-capillary DNA analyzer. Microsatellite Plugin in Geneious v.6
333 (Biomatters) was used to score alleles and loci were distinguished based on expected size range. To examine
334 relationships among all isolates and isolates from different environments, a minimum spanning network was
335 constructed using the Bruvo's genetic distance model[33] in the Poppr package executed in R[34].
336
337 ***Library preparation and whole genome sequencing***
338 Genomic DNA was sheared to a mean size of 600 bp using a Covaris LE220 focused ultrasonicator (Covaris Inc.,
339 Woburn, MA). DNA fragments were Ampure (Beckman Coulter Inc., Indianapolis, IN) cleaned and used to prepare
340 dual-indexed sequencing libraries using NEBNext Ultra library prep reagents (New England Biolabs Inc., Ipswich,
341 MA) and barcoding indices synthesized in the CDC Biotechnology Core Facility. Libraries were analyzed for size
342 and concentration, pooled and denatured for loading onto flowcell for cluster generation. Sequencing was performed
343 on an Illumina Hiseq using 300×300 cycle paired-end sequencing kit. On completion, sequence reads were filtered
344 for read quality, base called and demultiplexed using Casava v1.8.4. All raw reads and assemblies were deposited in
345 GenBank under project #XXXXXX.
346
347 ***SNP calling and neighbor-joining tree***
348 Cleaned whole genome sequence reads for each isolate were *de novo* assembled using SPAdes v3.12.0[35] with option
349 --careful and trained to Af293 reference genome[36] using option --trusted-contigs. Corrected fasta files generated from
350 SPAdes were aligned to Af293 reference genome using Burrows-Wheeler Aligner (BWA) alignment tool[37] and
351 duplicate reads were marked using Picard v2.16.0. Single nucleotide polymorphisms (SNPs) were called with
352 SAMtools mpileup v1.6[37] with option -I to exclude insertions and deletions then with BCFtools v1.9 with option –c.
353 Bases with phred quality score lower than 40 were filtered using SAMtools seqtk v1.2. SNP data were converted into
354 interleaved phylip format and a neighbor-joining tree was constructed using Seaview v4.7[38]. Support for internal
355 branches was determined by 100 bootstrap replicates. The tree was visualized and annotated using iTOL:
356 International Tree of Life v5[39].
357
358
359

Genome mining for agricultural fungicide resistance

Whole genome sequences (Supplementary Table S3) were searched by tblastn[40] for *A. fumigatus cyp51A* (GenBank accession number EDP50065), *benA* (GenBank accession number EDP56324), and *cytB* (GenBank accession number AFE02831). Blast hits were extracted from assemblies using BEDtools v2.26.0. Sequence analysis was performed using Geneious v11.1.5 (Biomatters, Auckland, NZ).

Fungicide resistance phenotype assays

Sensitivity assays for QoI were conducted in 100-mm Petri dishes of Sabouraud dextrose agar (SDA) that contained 10 µg/ml of azoxystrobin (Sigma Aldrich analytical-grade, diluted from 10 mg/mL stock in acetone) and 100 µg/ml salicylhydroxamic acid (SHAM) (Sigma Aldrich analytical-grade, diluted from 100 mg/ml stock in methanol)[41]. Controls were identical except that they lacked azoxystrobin. Sensitivity assays for MBC fungicides were similar, except they contained only 10 µg/ml benomyl (Sigma Aldrich, diluted from10 mg/ml stock in DMSO) in SDA[42]. Controls lacked Benomyl. Three azoxystrobin-amended SDA and three control dishes, as well as 3 benomyl-amended and control dishes were inoculated with 100 µl of a 1×10^3 conidia/ml *A. fumigatus* stock, spread using a sterile glass rod, and incubated at 37°C for 23 h at which point microcolonies were counted by eye. The experiment was performed twice.

References (for Methods)

29 CLSI. *Reference method for broth dilution antifungal susceptibility testing of filamentous fungi; approved standard*. Vol. CLSI document M38-A2. (2008).

30 Momany, M., Westfall, P. J. & Abramowsky, G. Aspergillus nidulans swo mutants show defects in polarity establishment, polarity maintenance and hyphal morphogenesis. *Genetics* **151**, 557-567 (1999).

31 Pitkin, J. W., Panaccione, D. G. & Walton, J. D. A putative cyclic peptide efflux pump encoded by the TOXA gene of the plant-pathogenic fungus Cochliobolus carbonum. *Microbiology* **142 (Pt 6)**, 1557-1565, doi:10.1099/13500872-142-6-1557 (1996).

32 Mellado, E., Diaz-Guerra, T. M., Cuenca-Estrella, M. & Rodriguez-Tudela, J. L. Identification of two different 14-alpha sterol demethylase-related genes (cyp51A and cyp51B) in Aspergillus fumigatus and other Aspergillus species. *J Clin Microbiol* **39**, 2431-2438, doi:10.1128/JCM.39.7.2431-2438.2001 (2001).

33 Bruvo, R., Michiels, N. K., D'Souza, T. G. & Schulenburg, H. A simple method for the calculation of microsatellite genotype distances irrespective of ploidy level. *Mol Ecol* **13**, 2101-2106, doi:10.1111/j.1365-294X.2004.02209.x (2004).

34 Kamvar, Z. N., Tabima, J. F. & Grunwald, N. J. Poppr: an R package for genetic analysis of populations with clonal, partially clonal, and/or sexual reproduction. *PeerJ* **2**, e281, doi:10.7717/peerj.281 (2014).

35 Nurk, S. Assembling Genomes and Mini-metagenomes from Highly Chimeric Reads in *Research in Computational Molecular Biology. RECOMB 2013. Lecture Notes in Computer Science* Vol. 7821 (eds M. Deng, R. Jiang, F. Sun, & X. Zhang) (Springer, 2013).

36 Nierman, W. C. *et al.* Genomic sequence of the pathogenic and allergenic filamentous fungus Aspergillus fumigatus. *Nature* **438**, 1151-1156, doi:10.1038/nature04332 (2005).

37 Li, H. *et al.* The Sequence Alignment/Map format and SAMtools. *Bioinformatics* **25**, 2078-2079, doi:10.1093/bioinformatics/btp352 (2009).

38 Gouy, M., Guindon, S. & Gascuel, O. SeaView version 4: A multiplatform graphical user interface for sequence alignment and phylogenetic tree building. *Mol Biol Evol* **27**, 221-224, doi:10.1093/molbev/msp259 (2010).

39 Letunic, I. & Bork, P. Interactive Tree Of Life (iTOL) v4: recent updates and new developments. *Nucleic Acids Res* **47**, W256-W259, doi:10.1093/nar/gkz239 (2019).

40 Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. Basic local alignment search tool. *J Mol Biol* **215**, 403-410, doi:10.1016/S0022-2836(05)80360-2 (1990).

41 Vega, B., Liberti, D., Harmon, P. F. & Dewdney, M. M. A Rapid Resazurin-Based Microtiter Assay to Evaluate QoI Sensitivity for Alternaria alternata Isolates and Their Molecular Characterization. *Plant Dis* **96**, 1262-1270, doi:10.1094/PDIS-12-11-1037-RE (2012).

42 Summerbell, R. C. The benomyl test as a fundamental diagnostic method for medical mycology. *J Clin Microbiol* **31**, 572-577 (1993)

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Author Contributions

S.E.K., M.T.B., and M.M. conceived and designed the research. S.E.K., T.M., L.G.S., and B.M. conducted the experiments. S.E.K., M.T.B., and L.G.S. collected environmental samples. S.E.K., T.M., L.G.S., B.M., M.M. and M.T.B analyzed the data. S.E.K., M.M. and M.T.B. wrote the manuscript with contributions from all authors.

Competing interests The authors declare no competing interests.

Materials requests & Correspondence should be addressed to M.T.B. or M.M.

433 **SUPPLEMENTARY TABLE S1. Sampling of *A. fumigatus* strains from agricultural sites**

Crop and/or substrate sampled[a]	Location(s) sampled	Sampling date(s) MM/DD/YY (no. sites[b])	Isolates collected, no. grew on TEB-amended plates	MIC[c]		WGS strains[d] (*azole-resistant)	No. isolates genotyped at *cyp51A* & STR*4f*[e]
				Sensitive	Resistant (MIC values)		
Apple (soil & plant debris)	Union Co., GA	08/21/17 (1)	9, 4	5	0	eAF76, eAF77	5
Blueberry (soil & plant debris)	Clinch Co., GA Bacon Co., GA	01/17/18 (3) 01/18/18 (2)	1, 0 16, 0	1 2	0 0	eAF584	0 1
Citrus (soil & plant debris)	Polk Co., FL	03/28/18 (13)	64, 8	11	0	eAF735, eAF743, eAF749, eAF768, eAF770, eAF773, eAF776, eAF777, eAF790, eAF792	10
Watermelon (soil & plant debris)	Crisp Co., GA Hillsborough Co., FL	06/07/17 (1) 03/27/18 (1)	3, 0 2, 2	1 1	0 0	eAF1 eAF740	1 1
Watermelon (soil & plant debris)	Crisp Co., GA	07/07/17 (1) 08/01/17 (1) 08/27/17 (1)	0, 0 18, 7 34, 24	0 9 25	0 0 0	eAF6, eAF10, eAF15, eAF16, eAF17, eAF39, eAF43	0 9 25
Compost pile	Clarke Co., GA	12/06/17 (1)	22, 12	2	11 (1 TEB & VOR = 16 µg/mL; 10 TEB & VOR > 16 µg/mL)	eAF222*, eAF223, eAF227, eAF233*, eAF234*	13
Grape (soil & plant debris)	Union Co., GA	08/21/17 (1)	14, 7	9	0	eAF21, eAF22, eAF61, eAF67, eAF72	9
Tomato (soil & plant debris)	Hillsborough Co., FL	03/27/18 (3)	35, 3	7	0	eAF620, eAF621, eAF623, eAF624, eAF625, eAF645, eAF647	7
Strawberry (soil & plant debris)	Hillsborough Co., FL	03/27/18 (1)	25, 3	5	1 (TEB = 4 µg/mL)	eAF586, eAF587, eAF589, eAF591, eAF609*, eAF610	6
Peanut (soil & plant debris)	Tift Co., GA	10/20/17 (6)	28, 18	22	0	eAF90, eAF91, eAF94, eAF95, eAF111,	22

						eAF113 eAF144, eAF148, eAF152, eAF153, eAF154,	
Peanut (plant debris)	Tift Co., GA Turner Co., GA	10/20/17 (4) 10/20/17 (1)	59, 27 4, 2	33 3	0 0	eAF81, eAF98, eAF100, eAF105, eAF116, eAF122, eAF128, eAF146, eAF147, eAF155, eAF158, eAF163, eAF177, eAF170	32 3
Pecan (plant debris; two sites were processing facilities)	Dougherty Co., GA	12/13/17 (11)	294, 6	19	1 (TEB & VOR MIC > 16 µg/mL)	eAF263, eAF265, eAF272, eAF288, eAF321, eAF325, eAF365, eAF379, eAF406, eAF477, eAF490, eAF500, eAF513*, eAF514, eAF537, eAF544, eAF549, eAF554, eAF560	19
Pecan (soil & plant debris)	Dougherty Co., GA	12/13/17 (2)	28, 0	2	0	eAF294, eAF335	2
Organic cucurbits (soil & plant debris)	Oconee Co., GA	12/06/17 (1)	28, 0	1	0		0
Organic brassicas (soil & plant debris)	Clarke Co., GA	12/06/17 (1)	16, 0	1	0	eAF237	1
Total		56 sites	700, 123	159	13	92	166

[a]Four samples were collected from distinct locations at each site; soil & plant debris indicates that soil was sampled and decaying plant debris on the soil surface was included in the sample.

[b]Each site was defined as a different field location, different crop at the same field location, or different triazole fungicide treatment

[c]All strains that grew on the TEB-amended medium were assayed for MIC. Additionally, 1 or 2 strains per site that did not grow on the amended medium were assayed for MIC for comparison. Isolates were considered resistant if TEB, ITC or VOR MIC values were > 2 µg/mL, or POS > 1 µg/mL; otherwise they were considered sensitive. MIC

assays were conducted twice for all resistant isolates and most sensitive isolates. Most often, there was no variation between replicates, but if there was a difference, the greater MIC is presented here.

[d]For whole genome sequencing (WGS) and phylogenetic analyses, 1 strain that grew and 1 strain that did not grow on TEB-amended medium that had been assayed for MIC were analyzed per site. WGS from three isolates (eAF105, eAF263, eAF365) were not included in the NJ tree due to excessive missing data.

[e]*cyp51A* genotypes were analyzed from all WGS strains and *cyp51A* genotypes for all remaining strains that grew on TEB-amended medium were analyzed by PCR and Sanger sequencing. All strains were genotyped with STR*Af* microsatellite markers.

SUPPLEMENTARY TABLE S2. Minimum inhibitory concentrations (MIC)[a] for *A. fumigatus* (*n* = 172) isolated from agricultural environments in Georgia and Florida where azole fungicides were applied.

Azole	Final Drug Concentration (µg/mL)								
	>16	16	8	4	2	1	0.5	0.25	<0.25
Tebuconazole	11	1		1	85	68	6		
Itraconazole					11	140	21		
Voriconazole	12					1	81	72	6
Posaconazole						15	93	58	6

[a]Isolates were assayed for MIC once (some sensitive isolates) or twice (all resistant and some sensitive isolates). Most often, there was no variation between replicates, but if there was a difference, the greater MIC is presented here.

SUPPLEMENTARY TABLE S3. Publicly available genome sequence data used in this study for neighbor-joining tree and mining for antifungal-resistance genes and mutations (italics)

NCBI_SRA_ ID	Taxa_ID	Sampling Location	Environ (E) /Clinical (C)	Azole-Res (R) /Sens(S)	Reference
ERR769506	08-12-12-13	Netherlands	C	R	doi: 10.1128/mBio.00536-15
ERS663179	08-19-02-10	Netherlands	E	R	doi: 10.1128/mBio.00536-15
ERS663176	08-19-02-30	Netherlands	E	S	doi: 10.1128/mBio.00536-15
ERR769512	08-19-02-46	Netherlands	E	R	doi: 10.1128/mBio.00536-15
ERR769509	08-19-02-61	Netherlands	E	R	doi: 10.1128/mBio.00536-15
ERR769508	08-31-08-91	Netherlands	C	R	doi: 10.1128/mBio.00536-15
ERS663166	09-7500806	UK	C	S	doi: 10.1128/mBio.00536-15
ERR769511	10-01-02-27	Netherlands	C	R	doi: 10.1128/mBio.00536-15
ERS663168	12-7504462	UK	C	S	doi: 10.1128/mBio.00536-15
ERS663167	12-7504652	UK	C	S	doi: 10.1128/mBio.00536-15

ERS663169	12-7505054	UK	C	S	doi: 10.1128/mBio.00536-15
ERS663165	12-7505220	UK	C	R	doi: 10.1128/mBio.00536-15
ERS663164	12-7505446	UK	C	R	doi: 10.1128/mBio.00536-15
ERR232426	A1163	France	C	S	NCBI SRA
ERS663170	Af293	UK	C	S	doi: 10.1128/mBio.00536-15
ERX207014	AF300	UK	C	S	NCBI SRA
SRS375791	AF41	UK	C	S	NCBI SRA
ERS663171	AF65	UK	C	S	doi: 10.1128/mBio.00536-15
SRR617721	AF72	UK	C	R	doi: 10.1128/AAC.41.6.1364
SRR617722	AF90	UK	E	R	doi: 10.1128/AAC.41.6.1364
ERS663181	Afu_1042/09	India	C	R	doi: 10.1128/mBio.00536-15
ERS663184	Afu_124/E11	India	E	R	doi: 10.1128/mBio.00536-15
ERS663185	Afu_166/E11	India	E	R	doi: 10.1128/mBio.00536-15
ERS663187	Afu_218/E11	India	E	R	doi: 10.1128/mBio.00536-15
ERS663186	Afu_257/E11	India	E	R	doi: 10.1128/mBio.00536-15
ERS663183	Afu_591/12	India	C	R	doi: 10.1128/mBio.00536-15
ERS663180	Afu_942/09	India	C	R	doi: 10.1128/mBio.00536-15
ERS216929	AP65	UK	E	S	NCBI SRA
SRR7418943	ATCC_204305	USA	C	S	doi: 10.3390/genes9070363
SRR7418935	ATCC_46645	UK	C	S	doi: 10.3390/genes9070363
ERS216946	CF098	UK	C	S	NCBI SRA
ERR232405	CF337	UK	C	R	NCBI SRA
SRR7418947	CM2141	Spain	C	S	doi: 10.3390/genes9070363
SRR7418942	CM237	Spain	C	S	doi: 10.3390/genes9070363
SRR7418945	CM3248	Spain	C	S	doi: 10.3390/genes9070363
SRR7418944	CM5419	Spain	C	S	doi: 10.3390/genes9070363
SRR7418949	CM5757	Spain	C	S	doi: 10.3390/genes9070363
SRR7418936	CM6458	Spain	C	S	doi: 10.3390/genes9070363
SRR7418938	CM7555	Spain	C	R	doi: 10.3390/genes9070363
SRR7418941	CM7632	Spain	C	S	doi: 10.3390/genes9070363
ERR232430	D17	UK	E	R	NCBI SRA
SRR617724	F12219	UK	C	R	doi: 10.3201/eid1507.090043
SRR617725	F12636	UK	C	R	doi: 10.3201/eid1507.090043
SRR617727	F13619	UK	C	R	doi: 10.3201/eid1507.090043
SRR617729	F14403	UK	C	R	doi: 10.3201/eid1507.090043

SRR617731	F14532	UK	C	R	doi: 10.3201/eid1507.090043
SRS375803	F14946G	UK	C	S	doi: 10.3201/eid1507.090043
SRR617733	F15390	UK	C	R	doi: 10.3201/eid1507.090043
SRR617735	F16134	UK	C	R	doi: 10.3201/eid1507.090043
SRS375808	F16311	UK	C	S	doi: 10.3201/eid1507.090043
ERR232417	F17999	UK	C	R	doi: 10.3201/eid1507.090043
SRS375813	F18085	UK	C	S	doi: 10.3201/eid1507.090043
ERR232439	F18304	UK	C	R	doi: 10.3201/eid1507.090043
ERR232418	F18329	UK	C	R	doi: 10.3201/eid1507.090043
ERR232413	F20140	UK	C	R	doi: 10.3201/eid1507.090043
ERR232440	F21572	UK	C	R	doi: 10.3201/eid1507.090043
ERR232414	F21732	UK	C	R	doi: 10.3201/eid1507.090043
ERR232421	F21857	UK	C	R	doi: 10.3201/eid1507.090043
ERS216942	F4S1B	UK	E	S	NCBI SRA
SRS375812	F5211G	UK	C	S	doi: 10.3201/eid1507.090043
SRS375815	F7763	UK	C	S	doi: 10.3201/eid1507.090043
SRR4002443	ISSFT-021	International Space Station	E	S	doi: 10.1128/mSphere.00227-16
ERR232441	RSF2S8	UK	E	R	NCBI SRA
ERS216922	SF1S5	UK	E	S	NCBI SRA
ERS216948	SF1S6	UK	E	S	NCBI SRA
ERS216938	SF2S6	UK	E	S	NCBI SRA
ERS216937	SF3S1	UK	E	S	NCBI SRA
ERS216930	SF3S10	UK	E	S	NCBI SRA
ERS216918	SF4S10	UK	E	S	NCBI SRA
ERR769507	*08-36-03-25*	*Netherlands*	*C*	*R*	*doi: 10.1128/mBio.00536-15*
ERS663182	*Afu_343/P/11*	*India*	*C*	*R*	*doi: 10.1128/mBio.00536-15*
SRR7841978	*DI_15-102*	*USA*	*C*	*R*	*doi: 10.1128/mBio.00437-20*
SRR7841983	*DI_15-106*	*USA*	*C*	*R*	*doi: 10.1128/mBio.00437-23*
SRR7841993	*DI_15-96*	*USA*	*C*	*R*	*doi: 10.1128/mBio.00437-36*
SRR7842000	*DI_15-116*	*USA*	*C*	*R*	*doi: 10.1128/mBio.00437-31*

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SUPPLEMENTARY TABLE S4. Fungicide resistance genotypes and growth phenotypes for agricultural isolates from Georgia and Florida.

Isolate	*cyp51A* genotype	Growth on Azole[a]	*cytB* genotype	Growth on QoI[b]	*benA* genotype	Growth on MBC[c]
eAF77	WT	-	WT	-	WT	-
eAF94	WT	-	WT	-	WT	-
eAF128	WT	-	WT	-	WT	-
eAF537	WT	-	WT	-	WT	-
eAF222	TR_{46}/Y121F/T289A	+	G143A	+	F219Y	+
eAF233	TR_{46}/Y121F/T289A	+	G143A	+	F219Y	+
eAF234	TR_{46}/Y121F/T289A	+	G143A	+	F219Y	+
eAF513	TR_{46}/Y121F/T289A	+	G143A	+	F219Y	+

[a] One hundred conidia were inoculated to solid medium containing 3 μg/ml tebuconazole (TEB) and incubated at 37°C for 23-24 h. These isolates also had MICs $\geq$16 μg/ml in TEB liquid medium (Table 1).

[b] One hundred conidia were inoculated to solid medium containing 10 μg/ml azoxystobin and incubated at 37°C for 23-24 h.

[c] One hundred conidia were inoculated to solid medium containing 10 μg/ml benomyl and incubated at 37°C for 23-24 h.

474 **Supplementary Figure S1**
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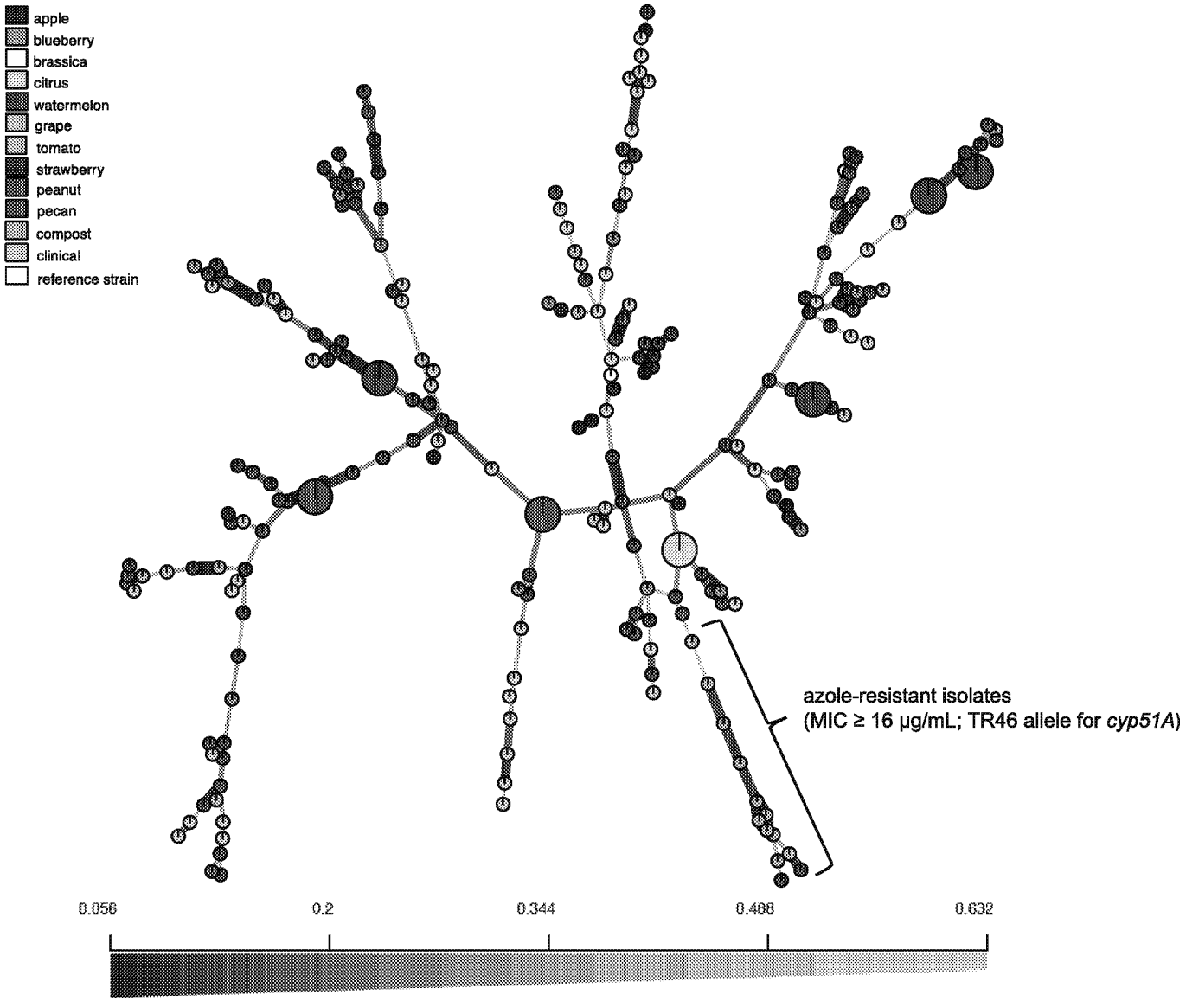

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479 **Supplementary Figure S1.** Minimum spanning network based on Bruvo's genetic distance of agricultural and
480 clinical isolates of *A. fumigatus* from Georgia and Florida. Isolates (168 agricultural and 48 clinical) were genotyped
481 with 9 STR*Af* markers. Each circle represents a unique haplotype and the size of the circle represents the relative
482 frequency of detection. The color of each circle represents the environment where the isolate was collected. Thicker
483 lines represent shorter genetic distances. Individuals with the TR46/Y121F/T289A allele for *cyp51A* are shown in the
484 lower right.

485 **Supplementary Figure S2**
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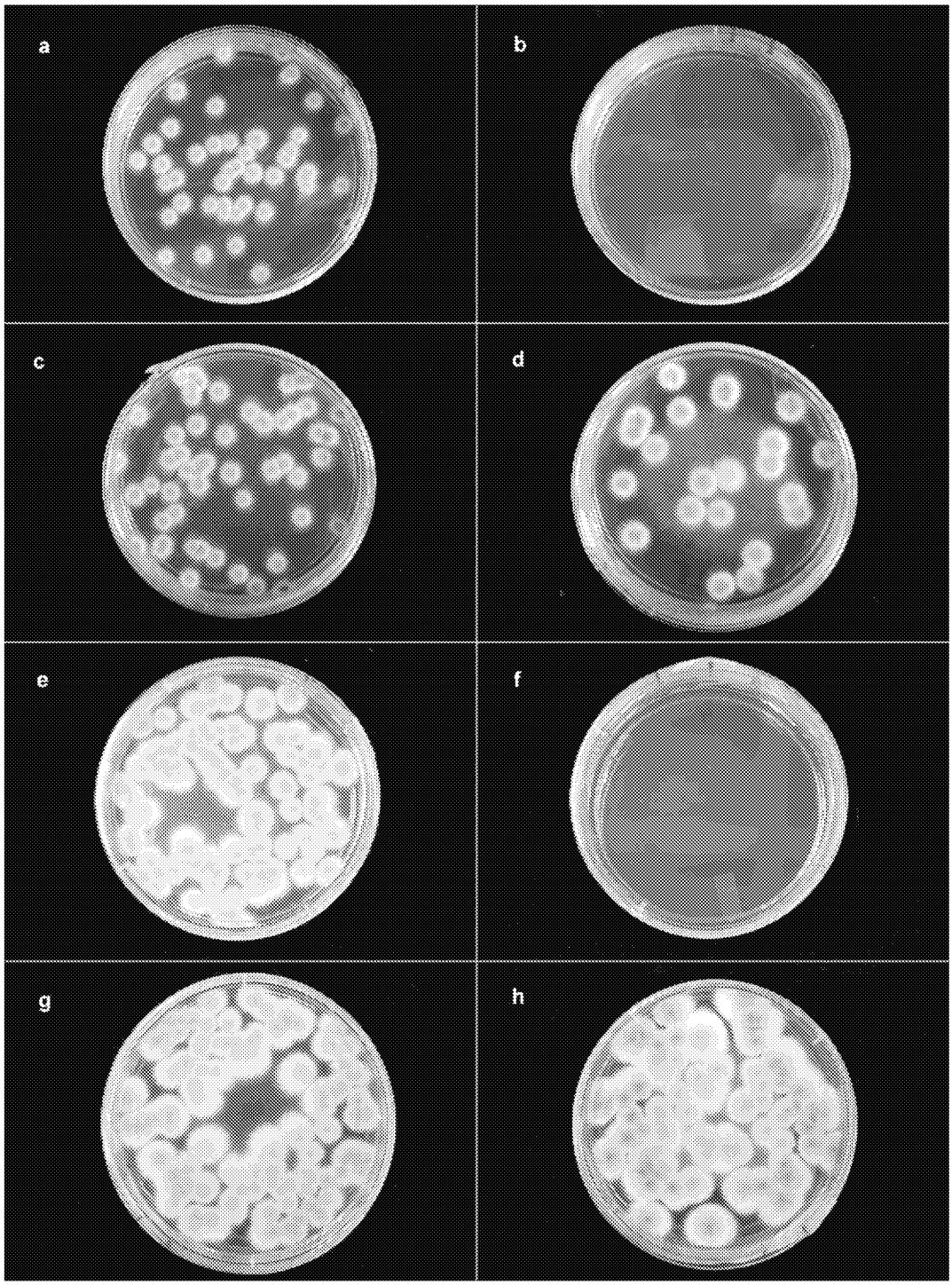

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490 **Supplementary Figure S2.** Pan-azole-resistant *A. fumigatus* (*cyp51A* TR_{46}/Y121F/T289A) with *cytB* G143A and
491 *benA* F219Y mutations are resistant to quinone outside inhibitor (QoI) and benzimidazole (MBC) fungicides. Left
492 column (a, c, e, g) multi-fungicide-resistant isolate eAF222. Right column (b, d, f, h) sensitive isolate eAF94. (a, b)
493 SDA (Sabouraud dextrose agar) + the QoI fungicide azoxystrobin + salicylhydroxamic acid (SHAM). (c, d) SDA
494 medium + SHAM. (e,f) SDA medium + the MBC fungicide benomyl. (g, h) SDA medium.